

Isolation of Skin Test-Active Preparations from Yeast-Phase Cells of *Blastomyces dermatitidis*

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Cell wall and cytoplasmic fractions were isolated from mechanically disrupted yeast-phase cells of *Blastomyces dermatitidis* in an effort to obtain a reliable skin-test antigen. The biological activities of these fractions were compared with those of two blastomycin preparations. The cytoplasmic antigens exhibited a low index of specificity yet exceeded the specificities of the blastomycins. The skin test-active component(s) of the cytoplasmic material had a molecular weight between 10,000 and 30,000 and could easily be concentrated by ultrafiltration on a PM-10 membrane. Unlike the cytoplasmic antigens and blastomycins, an alkali-soluble, water-soluble cell wall antigen effectively distinguished guinea pigs that were sensitized to *B. dermatitidis* from those sensitized to *Histoplasma capsulatum*. The biological activity of the yeast-phase antigen could be quantitated, on a weight basis, after purification by ultrafiltration (PM-10 membrane).

It is well established that blastomycin preparations, the broth culture filtrates of mycelial-phase cells of *Blastomyces dermatitidis*, lack sensitivity or specificity, or both. Repeated studies have shown that patients with histoplasmosis commonly react to blastomycins although only 40 to 50%, or even less, of patients with proved blastomycosis react. Previous attempts to obtain a reliable skin-test antigen, i.e., one which exhibits sensitivity and specificity, have for the most part been limited to analyses of broth culture filtrates obtained from mycelial- or yeast-phase cells (2, 3). However, recovery of a skin test-active preparation from culture filtrates does not necessarily lend itself to either quantitative or qualitative reproduction. An alternative approach, then, would be to ascertain which fungal constituent(s) elicits a delayed-type hypersensitivity response in blastomycosis and to develop procedures for isolating purified preparations of the antigen.

To initiate these studies, we isolated cell wall and cytoplasmic fractions from yeast-phase cells of *B. dermatitidis* and compared the skin test reactivity of these fractions with that of two blastomycin preparations.

MATERIALS AND METHODS

Cultures. *B. dermatitidis* strain SCB-2 (ATCC 26199) was isolated from a human case of blastomycosis and made available by Arthur Di Salvo, South

Carolina State Health Dept., Columbia, S.C. The human isolate (Scratchfield) of *Histoplasma capsulatum* was obtained from the Missouri State Chest Hospital, Mt. Vernon, Mo. Stock cultures of these two organisms were maintained on brain heart infusion agar (Difco, Detroit, Mich.) at 37 C.

Our procedure for obtaining cell wall and cytoplasmic fractions is outlined in Fig. 1. Yeast-phase cells, harvested from 3-day-old cultures on brain heart infusion agar slants, were inoculated into flasks containing brain heart infusion broth. The broth cultures were incubated at 37 C in a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) with constant shaking at 120 rpm. After 72 h of growth (log phase), merthiolate was added to a final concentration of 1:10,000, and the cultures were placed at 4 C for 24 h. The merthiolate-killed cells were harvested by centrifugation ($5,000 \times g$, 10 min) and then washed with distilled water to remove the bulk of medium constituents.

Cell wall fractionation. A detailed procedure for the isolation and purification of cell wall fractions has been reported previously (1). Briefly, yeast-phase cells were suspended with an equal volume of glass beads (0.45 to 0.55 mm in diameter) and then shaken in a Braun model MSK homogenizer for 90 s at 2,000 rpm. A carbon dioxide cooling device was used to minimize heat effects during breakage. The homogenized cell suspension was centrifuged at $27,000 \times g$ for 10 min in a Sorvall model RC2-B refrigerated centrifuge. The supernate (crude cytoplasm) was decanted and saved for further study. The cell wall pellet was resuspended in distilled water and centrifuged and the supernate was discarded. This process was repeated five times.

Cell walls were partially purified of cytoplasmic

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constituents by incubation in 0.01 M potassium phosphate buffer (pH 7.2) containing trypsin (100 $\mu\text{g}/\text{mg}$). The trypsin-treated walls were hydrolyzed with 1 N NaOH (1 ml/mg) for 3 h in a water-bath shaker at 25 C. Afterwards, the suspension was centrifuged at $27,000 \times g$ for 10 min. The alkali-soluble supernate was decanted, passed through a membrane filter (0.45- μm pore size, Millipore Corp., Bedford, Mass.), then dialyzed against several changes of distilled water at 4 C (final pH 7.0). The alkali-soluble, water-insoluble glucan which precipitated during dialysis was removed by centrifugation. The supernate (alkali-soluble, water-soluble [ASWS]) was filtered and then lyophilized and stored at -20 C.

Cytoplasmic fractionation. The crude cytoplasm was first centrifuged at $27,000 \times g$ for 30 min and then passed through a membrane filter (0.45- μm pore size, Millipore Corp.). Cytoplasmic constituents having a molecular weight of approximately 100,000 or greater were removed by passing the suspension through a Diaflo XM-100 ultrafilter membrane (Amicon, Inc., Lexington, Mass.) under a nitrogen pressure of 24 lb/in². The XM-100 ultrafiltrate was lyophilized and then extracted with ethyl alcohol-diethyl ether (1:1, vol/vol) for 24 h at 25 C. The lipid-extracted residue was resuspended in and dialyzed against distilled water at 4 C for 48 h. To fractionate the cytoplasmic material into different molecular weight ranges, the XM-100 filtrate was passed through a series of Diaflo ultrafilter membranes (Fig. 1). The first, a XM-50 membrane, retains constituents having a molecular weight of 50,000 or greater. Likewise, the PM-30 and PM-10 membranes retain substances with molecular weights of approximately 30,000 and 10,000, respectively. After filtration, the membranes were reversed, and the residues were eluted in distilled water under a nitrogen pressure of 50 lb/in². The residues and the three ultrafiltrates were lyophilized and stored at -20 C until used.

Sensitization of guinea pigs. A total of 120 male Hartley inbred guinea pigs weighing 600 to 800 g were used during the course of this study: 55 were sensitized to yeast-phase cells of *B. dermatitidis* strain SCB-2, 55 were sensitized to yeast-phase cells of *H. capsulatum* (Scratchfield), and the remaining 10 (controls) were inoculated with saline only. Merthiolate-killed cells were suspended in sterile physiological saline to a concentration of 2×10^8 cells/ml, as estimated by hemocytometer counts. The cell suspension was emulsified with an equal volume of complete Freund adjuvant containing killed *Mycobacterium tuberculosis* H37Ra (Difco). Each guinea pig received 1 ml of the emulsion: 0.2 ml was injected into each of the two front footpads, and the remaining 0.6 ml was administered subcutaneously into the neck region. Skin tests were initiated 3 to 4 weeks after injection.

Skin tests. The cell wall and cytoplasmic antigens isolated from yeast-phase cells of *B. dermatitidis* strain SCB-2 were suspended in physiological saline to a concentration of 100 $\mu\text{g}/\text{ml}$ (dry weight). These preparations were filter-sterilized prior to skin tests. The biological activities of the yeast-phase antigens were compared with that of commercial blastomycin (1:25, Parke, Davis & Co., Detroit, Mich.), the

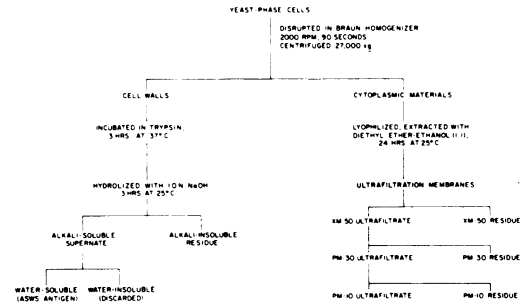


FIG. 1. Procedure for obtaining cell wall and cytoplasmic fractions from yeast-phase cells of *B. dermatitidis* strain SCB-2.

privately prepared mycelial-phase filtrate blastomycin KCB-25, diluted 1:25, and histoplasmin H-42 (1:25), obtained from the National Communicable Disease Center, Atlanta, Ga.

One-tenth milliliter of each preparation was injected intradermally into one of six skin-test sites on the shaved visceral surfaces of the guinea pigs. In most instances the yeast-phase antigens were tested simultaneously with the two blastomycin antigens. Indurations of 5 mm or greater in diameter, with or without erythema, were considered positive.

Specificity index. The yeast-phase antigens could not be compared accurately with the blastomycins on a weight basis. For this reason we have compared these antigens in terms of a specificity index (SI) or ratio of the number of positive reactors produced in the homologous group to those produced in the heterologous group. To minimize any variation in the heterologous group (*Histoplasma*-sensitized), only those guinea pigs which reacted to histoplasmin H-42 were used.

Histological sections. At 4, 24, and 48 h after skin testing, skin-test sites were excised from four *Blastomyces*-sensitized, four *Histoplasma*-sensitized, and two nonsensitized guinea pigs. The tissues were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin for histological evaluation.

RESULTS

Skin tests. The skin-test reactivity of the two reference blastomycins and that of histoplasmin H-42 are summarized in Table 1. Reactions to all three antigens were maximal 24 h after skin testing. Both commercial blastomycin and blastomycin KCB-25 elicited more reactions in the heterologously sensitized guinea pigs than in those sensitized to *B. dermatitidis*. Of the 54 *Blastomyces*-sensitized guinea pigs tested, only 20 (37%) reacted to commercial blastomycin, yet 12 (40%) of the 30 *Histoplasma*-sensitized animals also reacted. The increased sensitivity of blastomycin KCB-25 was accompanied by an increase in cross-reactivity, i.e., 66 and 74% of the *Blastomyces*- and *Histoplasma*-sensitized

guinea pigs, respectively, reacted to this antigen. The SI of the commercial antigen is 0.93, and that of blastomycin KCB-25 is 0.88. In sharp contrast to the blastomycins, histoplasmin H-42 elicited reactions in 96% of the *Histoplasma*-sensitized group, but only 13% of the heterologous group cross-reacted, and therefore exhibited a SI of 7.23.

Table 2 presents the results of skin tests of the sensitized guinea pigs with the yeast-phase cytoplasmic fractions. The XM-50 ultrafiltrate, containing constituents with a molecular weight of approximately 50,000 or less, elicited reactions in 40 of 45 or 89% of the *Blastomyces*-sensitized group at 24 h. Of these, 38 (84%) remained positive 48 h after skin testing. The

number of cross-reactions to the XM-50 ultrafiltrate was 63% at 24 h but diminished to only 11% 48 h after skin testing. Removal of constituents with a molecular weight of 30,000 to 50,000 decreased the SI of the cytoplasmic antigen. In fact, the number of homologous reactions (96%) elicited by the PM-30 ultrafiltrate approximated the number of cross-reactions (88%). The slight increase in activity of the PM-30 ultrafiltrate, in terms of the number of positive reactors (in both groups), is most likely attributable to the concentration effect of ultrafiltration. In other words, the active component(s) is probably more concentrated, on a weight basis, in the PM-30 ultrafiltrate than in the XM-50 fraction.

TABLE 1. Skin-test reactivity of blastomycins and histoplasmin H-42 in sensitized guinea pigs

Mycelial-phase antigen ^a	Blastomyces-sensitized		Histoplasma-sensitized	
	Induration (mm)		Induration (mm)	
	24 h	48 h	24 h	48 h
Commercial blastomycin	20/54 (37.0%) 9.1 ^b	6/54 (11.1%) 8.8	12/30 (40.0%) 9.3	2/30 (6.7%) 6.5
Blastomycin KCB-25	36/55 (65.5%) 10.8	19/55 (34.5%) 8.3	23/31 (74.2%) 14.9	13/31 (41.9%) 12.0
Histoplasmin H-42	6/45 (13.3%) 7.3	3/45 (6.7%) 9.3	50/52 (96.2%) 11.2	45/52 (86.5%) 10.2

^a Each guinea pig was inoculated intradermally with 0.1 ml of antigen (diluted 1:25).

^b Expressed as the mean induration (mm) of positive reactors only.

TABLE 2. Skin-test reactivity of yeast-phase cytoplasmic fractions of *B. dermatitidis* strain SCB-2 in sensitized guinea pigs

Cytoplasmic fraction ^a	Blastomyces-sensitized		Histoplasma-sensitized	
	Induration (mm)		Induration (mm)	
	24 h	48 h	24 h	48 h
Ultrafiltrates: XM-50	40/45 (88.9%) 12.9 ^b	38/45 (84.4%) 9.9	22/35 (62.9%) 9.3	4/35 (11.4%) 10.6
PM-30	26/27 (96.3%) 10.0	24/27 (88.8%) 8.1	15/17 (88.2%) 9.4	4/17 (23.5%) 8.6
PM-10	3/29 (10.3%) 6.8	1/29 (3.4%) 10.0	2/17 (11.8%) 5.5	0/17 (0.0%)
Membrane residue: PM-10	23/28 (82.1%) 12.8	19/28 (67.9%) 11.0	6/14 (42.9%) 8.8	3/14 (21.4%) 6.7

^a Each guinea pig was inoculated intradermally with 0.1 ml of antigen suspended in physiological saline (100 µg/0.1 ml). The values depicted are the averages of skin tests with two or more separate preparations of each antigen.

^b Expressed as the mean induration (mm) of positive reactors only.

Ultrafiltration of the PM-30 cytoplasmic fraction through a PM-10 membrane resulted in an almost complete loss in skin-test reactivity (Table 2). Of the 29 homologously sensitized guinea pigs that were skin-tested, only three (10%) reacted. Likewise, only two (12%) of the heterologous group reacted to the PM-10 ultrafiltrate. The inability of this fraction to elicit skin-test reactions suggested that the active component(s) was retained on the PM-10 ultrafilter membrane and would therefore have a molecular weight range of 10,000 to 30,000. Once eluted, this fraction (PM-10 residue) elicited reactions in 82 and 43% of the *Blastomyces*- and *Histoplasma*-sensitized guinea pigs, respectively. Although the SI of the PM-10 residue is only 1.90, it is twice that obtained with the blastomycins.

Of the various fractions that we have isolated from yeast-phase cell walls (1), only the ASWS fraction exhibited skin-test reactivity. As shown in Table 3, 38 (69%) of the 55 *Blastomyces*-sensitized guinea pigs reacted to the antigen at 24 h, whereas only four (8%) of the 51 *Histoplasma*-sensitized guinea pigs reacted. Although these results were encouraging, we found that the skin-test reactivity of the ASWS antigen varied from preparation to preparation. Since such differences were most likely attributable to differences in concentration of the active material, we sought to further purify the cell wall antigen. After ultrafiltration with a PM-10 membrane, we found that the reactivity was retained on the membrane. Of the 55 *Blastomyces*-sensitized guinea pigs skin-tested, 43 (78%) were positive reactors at 24 h, and 31 of the 55 exhibited positive reactions 48 h after

skin testing. On the other hand, only two of the 28 *Blastomyces*-sensitized guinea pigs and none of the 16 *Histoplasma*-sensitized guinea pigs reacted to the PM-10 filtrate obtained from the ASWS antigen. The SI of the ASWS antigen after ultrafiltration was 7.58, which is comparable to that (7.23) we observed for histoplasmin H-42. In addition to the increased sensitivity of the cell wall antigen after ultrafiltration, it became evident that the skin-test reactivity of the PM-10 residue was consistent from preparation to preparation. In a comparison of three separate preparations, a standard deviation of only 5.6% was obtained in the *Blastomyces*-sensitized guinea pigs. Prior to ultrafiltration, the number of positive reactors deviated 17.0%.

The ultrafiltrate fractionation was extended to include two additional Diaflo membranes, a PM-30 and a UM-20E, which retain molecular weight constituents of approximately 30,000 and 20,000, respectively. Unlike the effective separation achieved with the PM-10 membrane (with respect to the skin-test reactivity of the two fractions), no significant differences were observed in the reactivity of the fractions obtained from the PM-30 or UM-20E membranes. The PM-30 residue (100 μ g) elicited reactions in 77.7% of the *Blastomyces*-sensitized animals, and the PM-30 filtrate elicited reactions in 71.4% of the same animals. Similarly, reactions were produced in 77.8 and 83.3% of the *Blastomyces*-sensitized guinea pigs that were skin tested with the UM-20E residue and filtrate fractions, respectively.

The dose-response curve obtained when 26 *Blastomyces*-sensitized guinea pigs were skin-tested with 10, 50, and 100 μ g of the ASWS

TABLE 3. Skin-test reactivity of the ASWS cell wall fractions of *B. dermatitidis* strain SCB-2 in sensitized guinea pigs

Antigen ^a	<i>Blastomyces</i> -sensitized		<i>Histoplasma</i> -sensitized	
	Induration (mm)		Induration (mm)	
	24 h	48 h	24 h	48 h
ASWS	38/55 (69.1%) 10.5 ^b	22/55 (40.0%) 8.7	4/51 (7.8%) 7.6	1/51 (2.0%) 7.0
ASWS ultrafiltrate fractions:				
PM-10 residue	43/55 (78.1%) 12.2	31/55 (56.3%) 9.7	3/29 (10.3%) 10.3	0/29 (0.0%)
PM-10 filtrate	2/28 (7.1%) 7.5	0/28 (0.0%)	0/16 (0.0%)	0/16 (0.0%)

^a Each guinea pig was inoculated intradermally with 0.1 ml of antigen suspended in physiological saline (100 μ g/0.1 ml). The values depicted are the averages of skin tests with three separate preparations of each antigen.

^b Expressed as the mean induration (mm) of positive reactors only.

(PM-10 residue) is depicted in Fig. 2. To minimize any variation which might be attributed to the area inoculated, the sites were alternated for each of the three concentrations. The low dose (10 μ g) elicited reactions in six (23%) of the guinea pigs at 24 h, all of which were negative by 48 h. Twenty (76%) of the guinea pigs reacted to the 50- μ g dose by 24 h, but the positive reactors had decreased to 23% by 48 h. The largest dose (100 μ g) produced reactions in 21 (81%) and 15 (58%) at 24 and 48 h. Doses greater than 100 μ g per 0.1 ml produced marked indurations that were often accompanied by necrosis; therefore, they were not used in this study.

Histological reactions. Tissue sections made of skin-test sites 4 h after intradermal inoculation of a *Blastomyces*-sensitized guinea pig with the ASWS antigen (100 μ g) showed a marked infiltration of polymorphonuclear leukocytes and a sparse mononuclear cell response (Fig. 3A). The skin-test sites of two additional *Blastomyces*-sensitized guinea pigs were excised 24 h after inoculation. These sections revealed that the cellular infiltration was more marked than that observed at 4 h and that the response was primarily of the mononuclear cell type. The cellular infiltrate occurred deep within the dermis, notably around blood vessels, and often extended into muscle tissue (Fig. 3B). Sections obtained from a fourth guinea pig at 48 h showed that the mononuclear cell still predominated, although the extent of both polymorphonuclear and mononuclear infiltration had decreased from that observed at 24 h. In no instance did we detect any hemorrhagic or necrotic lesions in animals inoculated with 100

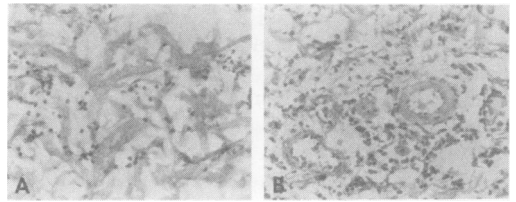


FIG. 3. Histological sections of skin-test sites excised from *Blastomyces*-sensitized guinea pigs at 4 h (A) and 24 h (B) after intradermal inoculation of the ASWS cell wall antigen (100 μ g). (Hematoxylin and eosin; $\times 160$.)

μ g of the ASWS antigen. The skin test reactions, recorded just prior to autopsy, were 10, 12, and 8 mm (induration) at 4, 24, and 48 h, respectively.

A similar histological response was observed in sections made of the skin test sites (in the same *Blastomyces*-sensitized guinea pigs) inoculated with blastomycin KCB-25. The polymorphonuclear cell predominated at 4 h; the maximal cellular infiltration, comprised mostly of mononuclear cells, was present at 24 h; and by 48 h the reaction was for the most part limited to a mononuclear cell response. An induration of 7 mm was observed in the first guinea pig (autopsied at 4 h); the two guinea pigs which were autopsied at 24 h exhibited indurations of 11 and 13 mm; and the fourth guinea pig (autopsied at 48 h) exhibited an induration of 8 mm in diameter.

Histoplasmin H-42 also elicited an early induration (4 h) in guinea pigs which were sensitized to *H. capsulatum*. Histologically, these reactions were characterized by a polymorphonuclear cell response. The induration observed in homologously sensitized guinea pigs skin tested with histoplasmin H-42, blastomycin KCB-25, and the ASWS antigen would not, by definition, be attributed to a delayed-type hypersensitivity response but probably represent an Arthus reaction. However, the histology observed at 24 and 48 h is consistent with that of a tuberculin reaction in a sensitized animal.

Histological sections of nonsensitized (controls) guinea pigs skin-tested with the ASWS antigen showed only a mild inflammatory reaction. This is in agreement with our observation that the cell wall antigen did not elicit an induration reaction and, with only a rare exception, did this antigen elicit even an erythematous response in the nonsensitized guinea pigs.

DISCUSSION

Two blastomycin preparations were compared simultaneously with the skin-test reactiv-

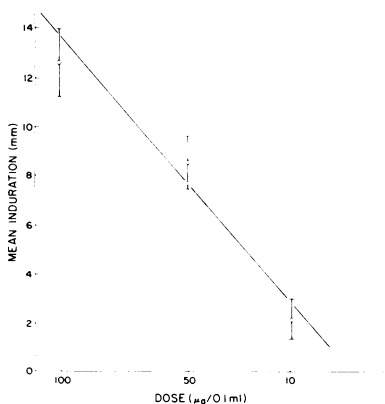


FIG. 2. Mean induration (mm) of skin test sites on 26 *Blastomyces*-sensitized guinea pigs 24 h after intradermal inoculation of 10- μ g, 50- μ g, and 100- μ g doses of the ASWS cell wall antigen. Vertical bars denote the standard error of induration.

ity of yeast-phase cell wall and cytoplasmic antigens. The blastomycins proved ineffective in distinguishing guinea pigs which were sensitized to yeast-phase cells of *B. dermatitidis* from those sensitized to *H. capsulatum*. With respect to each other, these mycelial-phase antigens differed only in the extent of their reactivity, i.e., blastomycin KCB-25 elicited a greater number of reactions in both groups. The SIs of the two antigens were comparable, which shows that the KCB-25 antigen is simply more concentrated than is the commercial preparation.

The most striking differences in the reactivity of the yeast-phase cytoplasmic ultrafiltrates and the blastomycins occurred 48 h after skin tests were performed. For example, 89 and 63% of the homologous and heterologous groups reacted to the XM-50 ultrafiltrate at 24 h. At 48 h, however, the number of cross-reactions had decreased to 11%, but the number of homologous reactions remained at 84%. Thus, the SI of the XM-50 ultrafiltrate had increased from 1.41 at 24 h to 7.4 at 48 h. In contrast, the specificity indexes of the blastomycins at 48 h did not increase appreciably from those obtained at 24 h.

The variability obtained in the skin-test reactivity of different preparations is a valid criticism of blastomycin antigens. We do not propose that the method used to obtain the cytoplasmic antigens will alleviate this problem. The skin test-active component(s) can be partially purified by the use of ultrafilter membranes, but, until further purification steps are developed, the PM-10 residue preparations would differ both quantitatively and qualitatively. Whether the component(s) which elicited the reactions in the *Blastomyces*-sensitized guinea pigs can be dissociated from the cross-reactive material or not was not ascertained in this study. However, the differences obtained in the SI values for the XM-50 ultrafiltrate (1.41), the PM-30 ultrafiltrate (1.09), and the PM-10 residue (1.90) support this possibility.

Of the yeast-phase fractions obtained in this study, the most promising antigen was the cell wall component liberated during alkaline hydrolysis. The greater specificity of this antigen as compared with those previously discussed is shown by the fact that at 24 h 78% of the *Blastomyces*-sensitized guinea pigs reacted, whereas only 10% of the *Histoplasma*-sensitized group reacted. Since this is the first indication that a cell wall component might prove useful as a skin-test antigen, many of our studies have been directed towards evaluating the efficacy of this antigen. Histologically, the ASWS antigen

elicited a tuberculin type of reaction in *Blastomyces*-sensitized guinea pigs at 24 and 48 h. Once purified by ultrafiltration (PM-10 membrane), the skin-test reactivity was reproducible within a 5% deviation; the obvious advantage of this is the possibility that the biological activity of the ASWS antigen can be standardized on a weight basis.

It should be mentioned that, in terms of the number of reactions elicited in the *Blastomyces*-sensitized guinea pigs, the ASWS antigen (100 μ g) was less reactive than were the cytoplasmic ultrafiltrates (100 μ g). This may be attributed to the greater sensitivity of the cytoplasmic component(s). Alternatively, the possibility exists that (i) the cytoplasmic antigens contained components that were nonspecific in their skin-test reactivity, or (ii) the native configuration of the cell wall antigen is altered during alkaline hydrolysis, thereby resulting in a decreased skin-test reactivity. The SIs of the cytoplasmic antigens tend to support the hypothesis that the reactions are, in part, non-specific.

Studies now in progress will compare the efficacy of the ASWS cell wall fractions obtained from additional strains of *B. dermatitidis*. Limited studies in this direction have been done previously (R. A. Cox, Ph.D. dissertation, Medical College of Georgia, Augusta, Ga., 1972), and the results were consistent with those reported here.

Whereas the results of this investigation have been most encouraging, we cannot, by inference, assume that the cell wall antigen will prove to be a reliable skin-test antigen in humans. It does, however, seem apparent that the results obtained in the guinea pig model warrant such an investigation.

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